Reviews

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ASPECTS OF GENE EXPRESSION ANALYSIS IN SMALL-VOLUME BLOOD SAMPLES USING THE REAL-TIME PCR

The gene expression profiling of peripheral blood leukocytes is a valuable method for assessing disease-associated and drug-response related genes. Small-volume blood samples can be used for gene expression analysis in biomarker discovery, pre-clinical animal research for drug development, neonatal and pediatric studies, and therapeutic drug monitoring. However, the reduced RNA quantity and handling procedures of small-volume samples complicate gene expression analysis using the quantitative real-time reverse transcription polymerase chain reaction. In this article, we systematized and compared the RNA isolation and molecular genetic techniques to disclose optimized strategies for the gene expression analysis of small-volume blood samples and discuss the gene expression studies in capillary blood samples.

Keywords: *small-volume blood sample, RT-qPCR, gene expression, capillary blood.*

Introduction

Leukocytes are one of the main components of blood and constitute an accessible source of clinically relevant data through specific reactions to the exposure to infectious agents, allergens, tumors, transplants, or autoimmune disorders [1—3]. The gene expression analysis of blood samples reflects differential patterns of gene regulation by assessing the matrix (m)RNA levels in the circulating leukocytes [1, 4—10].

In recent years, the reduction of analytical volumes of biological samples has played a crucial role in various biomedical domains [11—13], particu-

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larly in gene expression analysis [14—24]. For example, smaller blood volumes facilitate the development of test systems for gene expression analysis in capillary blood samples that can be conveniently collected from various sources (e.g., finger, earlobe tip, or heel) with inexpensive instruments. It can be used for disease diagnosis and health monitoring, particularly for children, patients with coagulation disorders, or in studies where multiple blood sampling is required to describe changes in biomarker levels over time [16, 17, 19]. This led to the need of development of optimized approaches to obtain RNA samples from micro volumes of whole blood without disturbing the transcript patterns induced by sampling, extraction, purification, or quantification methods.

Once RNA samples of sufficient quality and quantity have been obtained, gene expression analysis can be performed to identify differential patterns of gene regulation [1, 4—10]. Among the various methods available, quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is one of the primary approaches to process small-volume blood samples [1, 4—10]. However, several articles reported that gene expression analysis can be challenging due to the limited number of mRNAs in reduced-volume samples, resulting in misleading shifts of estimated mRNA levels [14, 17, 20].

This review summarizes the most common methods of RNA isolation and quantification for gene expression analysis in small-volume blood samples. It includes a discussion of various ways to optimize RNA isolation from small-volume blood samples, which can allow producing high-purity and integrity RNA and subsequent accurate and reproducible quantification data using RT-qPCR.

RNA Isolation Techniques from Small Volumes of Blood

Any quantitative gene expression analysis aims to isolate a sufficient amount of RNA, avoid its degradation and contamination (e.g., DNA that can lead to overestimation of transcript levels; inhibitors of the reverse transcription and qPCR steps; nucleases for long-term storage), and to facilitate the accurate determination of the pattern of genes expressed under specific circumstances [18, 20, 25—27]. *Table 1* lists the principles and advantages/disadvantages of the most popular methods used to isolate RNA.

Small- [20, 21], or sometimes low-volume [17, 22] blood samples are referred to the samples with significantly reduced volumes (generally ranging from 15 to 300 μL) compared with the traditional whole blood samples of 2.5 to 6 mL, which require peculiar methods of extraction of sufficiently concentrated, intact RNA and gene expression analysis [14—22]. RNA extraction from reduced sample volumes is associated with several complications, including higher susceptibility of small-volume samples to temperature fluctuations and delays in processing, resulting in RNA degradation, lower yields, and unwanted contamination (e.g., DNA, lysing agents, salts, protein, ethanol) and therefore impairing sample performance during gene expression analysis [14—22]. Organic extraction followed by spin-column or magnetic beads adsorption methods are currently the most efficient techniques for extracting sufficiently concentrated and intact RNA. They can be done by scaling down and optimizing standard procedures for total RNA isolation using commercial standardized kits [14—23, 28].

Several surfactant-based (tetradecyltrimethylammonium oxalate and tartaric acid in PAXgene Blood RNA System [14, 16, 20]) and chaotropic agent-based (guanidine hydrochloride in Tempus RNA stabilization reagent [10, 15, 17, 18, 21]; phenol and guanidine thiocyanate in TrioMol [19], TriFast [22] and QIAzol [23] reagents) methods were reported to be able to effectively lyse the cells as well as protect RNA from degradation by both endogenous found in blood and exogenous RNases [30]. Rinchai *et al.* stated vigorous mixing of blood samples with lysing agents (proportionally reduced in volume) immediately after collection as the crucial step for maximizing RNA yield during the extraction [15]. Speake *et al.* compared different mixing methods with 15 μL blood samples

when collected with a micropipette tube and found little impact on RNA quality when tubes were flicked (the RNA integrity number was an average of 7.6), pipetted (6.2) or vortexed (7.7), but was significantly reduced when samples were not mixed at all (4.3) [17]. Thorough homogenization, prolongation of incubation time with lysing agents [14, 20], and optimization of phase separation efficiency (by addition of chloroform when performing organic extraction [22, 23, 28]) are also beneficial.

Modifications of RNA purification include performing a DNase treatment step [16, 22], adding several RNA washing steps (using ice-cold isopropanol and methanol [22] or 75% ethanol [23]), dry spin [28], preheating [28] and using minimum effective (for operating equipment) volumes of the solvent.

Demonstrating the isolation of high-integrity total RNA from microlitre volumes of mouse whole blood Krawiec *et al.* suggested a proportional reduction of the buffer solution volumes

Table 1. **Characteristics of common RNA isolation methods**

Method	Principle	Advantages	Disadvantages	References
Organic extraction by the guanidinium-thiocyanate-phenol-chloroform / Trizol	2 phases are formed: the upper aqueous phase containing RNA, a ring of denatured proteins at the interphase, and the lower organic phase with cell debris	One of the most common methods; high RNA yield	It takes more time compared to other methods; the use of toxic organic solvents	[19, 22, 28]
Traditional salting-out methods	Selective RNA precipitation in a reaction mixture of high ionic strength, usually by lithium chloride (LiCl)	A fast and simple method for mRNA precipitation with minimal content of free nucleotides	It is not efficient for processing samples with low RNA concentration	[28]
Density gradient centrifugation	RNA migrates in a narrow density gradient made up by the dissociated solvent molecules (usually — cesium chloride (CsCl)) to the point with a similar density	It is used to isolate intact DNA-free RNA from various biological samples	The use of toxic solvents (CsCl) and mutagens (ethidium bromide (EtBr))	[28, 29]
Spin-column adsorption	RNA immobilization on the solid-phased spin-columns of different materials (e.g. silica, glass, or polyethylene fibers) under specific circumstances	One of the most common methods; takes less time; prevents cross-contamination; possibility of automation and optimization; high RNA yield	Additional steps to remove residual ethanol from the column before elution	[15, 18, 20, 21, 28]
Magnetic beads adsorption	RNA immobilization on the surface of magnetic particles in an external magnetic field	A fast environmentally friendly method; possibility of automation; high RNA yield	The surface of magnetic beads must be free from any contaminants; the magnetic field must be applied uniformly and be varied gradually	[17, 10, 28]

added to the spin-column or the magnetic beads system [14]. Thus an average of 2.3 μg of total RNA (or 50 μg for each mL of blood) was yielded from 50 μL of mouse blood when using the spin-column method [14]. However, the study of Robison *et al.* showed that using the standard volumes of solutions had no effect on the yields and was easier to employ [16]. Moreover, inappropriate volumes of working solutions can impair a sufficient binding to the column or magnetic beads and purification of target RNAs [15—18].

In the protocol of total RNA isolation from human fingerstick blood samples using the spin-column method, Robison *et al.* suggested using a SpeedVac centrifugal vacuum concentrator to concentrate the RNA volume to sufficient amounts for cDNA synthesis and qPCR [16]. In this manner, an average of 255.7 ng of total RNA (or 3.7 μg for each mL of blood) was extracted from 70 μL of whole blood that was collected by a fingerstick, whereas 6 μg of total RNA (or 2.4 μg for each mL of blood) was extracted from 2.5 mL of whole blood collected by traditional venipuncture samples [16]. However, this method can lead to considerable RNA degradation [31].

The noticeably lower yield of RNA from small-volume blood samples utilizing the methods by Robison *et al.* (3.7 μg for each mL of blood) [16] and Kalikiri *et al.* (11.6 μg (manual workflow) and 8.5 μg (automated workflow) for each mL of blood) [10] compared with the results obtained by Krawiec *et al.* (50 μg for each mL of blood) [14] and Fricano *et al.* (from 21.0 to 79.4 μg for each mL of blood) [24] most likely comes from the differences in mouse and human blood [32].

Because of RNAs instability *in vitro*, the additional steps of quantitative and qualitative assessment, in particular gel electrophoresis and spectrophotometry, respectively, are required at the end of any RNA isolation protocol [26]. While working with the low concentrations of the material analyzed and the limited sample volume that can be disposed of for the quality control measurement it is important to use equipment specifically designed for the analysis of low-concentration samples in micro volumes (e.g., microvolume spectrophotometer at the ultraviolet and visible light spectrum (UV-VIS) [10, 16, 19, 22], spectrofluorometer with a fluorescent RNA-binding dye, capillary gel electrophoresis system [18, 20], *etc.*).

Thus, RNA isolation from small volumes of blood can be challenged by the low content of RNAs in the samples and, therefore, it is necessary to modify the handling procedures [10, 14—22]. Several optimization points of spin-column and magnetic beard adsorption methods have been identified, maximizing the RNA yield and ensuring its quality and stability [10, 14—23, 28]. However, further systematic analysis is needed to develop a universal RNA isolation protocol reliable for small-volume blood samples. Comparative studies of developed protocols have to be performed to indicate the most effective steps in RNA isolation, and the working range of the volumes of blood samples needs to be established.

Molecular Genetic Techniques for Gene Expression Analysis of Small-Volume Blood Samples

The accuracy and reliability of gene expression analysis largely hinges on the suitability of the methods used for the analysis of samples of different analytical volumes.

RNA- or cDNA-microarrays are currently considered the best method for the large-scale (for example, the whole genome) experiments. The general principle is the hybridization of fluorescently labeled target molecules with oligonucleotide probes on the surface of the sensor [7, 8]. However, it was observed that microarray technology has a strict limitation of the working range of the template concentration in a reaction mixture. Moreover, Burgarner demonstrated that at low concentrations the equilibrium favors the absence of binding, which can lead to false-positive or false-negative test results [8].

The novel, highly efficient approach, such as RNA-sequencing, uses next-generation sequencing capabilities to detect and quantify a target

RNA [4, 10, 33]. However, it still requires further optimization due to technical difficulty, expense, reproducibility during mass production, and the absence of standardized protocols compared to other analysis methods [9, 10, 34, 35].

Currently, RT-qPCR remains the golden standard for quick, cheap, and simple gene expression analysis [7, 8]. Generally, in studies of new systems or drug targets, the key genes are identified by cDNA-microarray screening and then studied in more detail by more precise qPCR [5, 25, 36]. In addition, qPCR requires smaller amounts of mRNA samples and reagents and a shorter time for analysis [5, 6]. For example, Krawiec *et al.* used 50 μL of mouse whole blood to perform RT-qPCR and showed the expected dynamic and dose-dependent changes in the gene encoding heme oxygenase 1 in mice injected subcutaneously with CoPP, an inducer of heme oxygenase 1 in rodents [14]. In the study by Carrol *et al.* 300 μL of whole blood from pediatric patients was successfully analyzed using RT-qPCR [20]. The expression of genes encoding intercellular adhesion molecule 1 and interleukin 6 is increased in cases of invasive pneumococcal disease (such as meningitis and pneumonia) compared to healthy controls, and also is increased in survivors compared to non-survivors [20]. Thus RT-qPCR is a promising molecular genetic technique for gene expression analysis of small-volume blood samples.

Gene Expression Analysis in Small-Volume Blood Samples by RT-qPCR

The workflow of RT-qPCR consists of: cDNA synthesis from total RNA or mRNA samples using reverse transcriptase and cDNA amplification using thermostable DNA polymerase with real-time registration of the fluorescence signal [5, 37—40].

To the best of our knowledge no commonly preferred variations in RT-qPCR workflow or reaction mixture preparation are reported to optimize reverse transcription and qPCR protocols for small-volume blood samples. Although using the enzymes with high capacity, reduced RNase H activity (for reverse transcriptase), and increased thermostability can be advantageous for better performance in RNA samples of suboptimal concentration, purity, or integrity [14, 17, 41].

Both non-specific detection (e.g., SYBR Green [19] and EvaGreen [17, 21] dyes) and specific detection (via fluorescently labeled oligonucleotides, i.e. primers-probes, probes that emit fluorescent light during hydrolysis or hybridization with target DNA, and nucleic acids analogs [37, 40, 42—44]) [14, 20, 45] are used interchangeably in the studies of small-volume blood samples. However, due to the fluorescence of both amplified target DNA and reaction products, non-specific detection requires additional steps (e.g., melting curve analysis) to characterize the homogeneity of the amplicons [37, 42].

Due to the high sensitivity of modern qPCR assays and the inevitable presence of stochastic errors, the data normalization is required to remove the technical bias and unwanted variation. Using "housekeeping" genes, expressed at a relatively constant level, provides endogenous controls for effective RT-qPCR [46]. Carrol *et al.* studied 7 different housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β2 microglobulin, actin beta, hypoxanthine phosphoribosyltransferase, 60S ribosomal protein L14, succinate dehydrogenase complex flavoprotein subunit A, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta, and ribosomal protein L32) in 300 μL of blood and reported consistent expression level for all included assays [20]. These findings confirm that the analysis of small-volume blood samples can produce the reliable gene expression data across small-volume samples.

The study by Fricano *et al.* comparing the results of gene expression analysis of 9 high, mid, and low abundance genes (transcription factor 7, mitogen-activated protein kinase kinase 6, neuropilin 1, delta-aminolevulinate synthase 2, flavin adenine dinucleotide synthetase 1, calnexin, S100 calcium-binding protein A9, tumor necrosis factor, and interleukin-1 beta) during lipopolysac-

charide-induced acute immune response in rats obtained by RT-PCR and microarray analysis confirmed differences in the absolute fold change but the same trend across all abundance levels [24].

Overall, RT-qPCR presents a conventional method to detect the dynamic changes in gene expression patterns providing a versatile platform for small-volume blood processing.

Capillary Blood Gene Expression Analysis

Capillary blood testing is gaining attention as a minimally invasive alternative to venous blood sampling, particularly for point-of-care testing [47] and remote patient monitoring [48]. This method offers several advantages, including lower cost, less invasive, quick and easy procedure that doesn't require many technical skills, and increased capacity for repeated sampling, making it suitable for resource-limited settings and at-home testing [47—49]. Capillary blood sampling is commonly used for different analytical tests (i.e., biochemistry and hematology tests, such as glucose level, hemoglobin, and platelet count monitoring [50], infectious disease diagnostics [51], athlete biological passport [52], *etc.*) with results comparable to those from venous blood.

Physiologically, capillary blood differs from venous blood, reflecting their distinct roles in the circulatory system [53]. Capillary blood has different levels of proteins, enzymatic activity, electrolytes, and changes after glucose intake [49, 54—56]. Schalk *et al.* found a significant increase in leukocytes (3.5%) and red blood cell (2.2%) counts in capillary (fingerstick) blood samples compared to venous blood samples [57], which can contribute to altered gene expression depending on the sampling technique reported in several studies [16, 19]. Moreover, the age and medical condition of the subjects had a detectible influence on the differences between capillary and venous leucocyte values [57, 58].

A study by Robison *et al.* in 2009 comparing RNA isolated from blood samples from 70 μL of fingerstick and 2.5 mL venipuncture of samples registered the lowered synthesis of cDNA from RNA isolated from fingerstick blood (7.0 μg of cDNA from 50 ng of total RNA compared to 8.6 μg of cDNA in venipuncture sample) when the RNA quality assessed by spectrophotometry was identical [16]. Using GeneChip data Robison *et al.* found slightly higher average present calls, the percent of gene sequences on a given GeneChip called as present, for the venipuncture samples (56% compared to 52.5% for the capillary samples) [16]. Moreover, the fingerstick samples had higher 3'/5' ratios for *GAPDH* (8.7 compared to 2.8 for the venipuncture samples) and β-Actin (29.4 compared to 1.8 for the venipuncture samples) ratios on average [16], which verified the isolated RNA to be more susceptible to degradation [59]. Wehmeier and Hilberd in 2019 theorized further and suggested that some RNA degradation comes from the exposure of the capillary blood to oxygen before its transferal to the capillaries [19].

Futhermore, when comparing the expression values in smaller volumes to traditional ones, several studies found some differences [16, 19]. For example, studying the expression of the genes encoding the creatine kinases, cluster of differentiation 36 and the arginases 1 and 2 in capillary (20 μL) and venous (<200 μL) blood, Wehmeier and Hilberg observed lower ΔC_t values (C_t of the endogenous control *GAPDH* is deducted) of arginase 1 (3.55 in venous blood and 4.19 in capillary blood) and cluster of differentiation 36 (2.31 and 4.2, respectively) in venous blood and higher expression values of creatine kinases (1.11 and 0.13, respectively) compared to the capillary blood while arginase 2 remained identical (5.9 and 5.88, respectively) [19]. Whether these differences are based on a variable composition of blood cells in reduced-volume blood probes remains unclear. Overall, the shown differences were relatively weak and the data obtained matched well considering the analysis was done on relatively low-expressed targets (C_t values >30) [16, 19].

Therefore, the physiological features of capillary blood and the effect of microsampling on RNA isolation should be considered at the planning stage of

gene expression research. The detected changes in gene expression in capillary blood cannot be extrapolated to venous blood and vice versa.

Conclusions

Small-volume blood sampling is a commercially attractive method of collecting biological material, offering a promising basis to develop PCR assays for testing capillary or serial blood samples.

This review highlights the intricate nature of small-volume blood sample processing, underscoring the importance of the optimization of RNA extraction to evaluate the disease-associated genes.

While there are challenges associated with a limited number of mRNAs in the low-volume samples, resulting in misleading shifts of estimated mRNA levels, the use of appropriate strategies can produce isolated RNA of high purity and integrity and subsequent accurate and reproducible quantification data using RT-qPCR.

The physiological characteristics of capillary blood and the risks associated with sample collection and RNA isolation must be considered when determining the disease-associated genes in a patient's capillary blood.

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АСПЕКТИ АНАЛІЗУ ЕКСПРЕСІЇ ГЕНІВ У ЗРАЗКАХ
МАЛИХ ОБ'ЄМІВ КРОВІ МЕТОДОМ ПЛР В РЕАЛЬНОМУ ЧАСІ

Профілювання експресії генів лейкоцитів периферичної крові є важливим методом оцінки генів, пов'язаних із захворюваннями та реакцією на лікування. Зразки малих об'ємів крові можна використовувати для аналізу експресії генів під час пошуку біомаркерів, у доклінічних випробуваннях лікарських препаратів на тваринах, неонатальних і педіатричних дослідженнях та терапевтичному лікарському моніторингу. Однак зменшена кількість РНК та унікальні процедури обробки зразків малого об'єму ускладнюють аналіз експресії генів за допомогою полімеразної ланцюгової реакції зі зворотної транскрипції в реальному часі. У цій статті ми систематизуємо та порівнюємо методи виділення РНК і молекулярно-генетичні підходи для визначення оптимізованих стратегій аналізу експресії генів у зразках малих об'ємів крові та обговорюємо дослідження експресії генів у зразках капілярної крові.

***Ключові слова*:** зразок малого об'єму крові, ЗТ-ПЛР у реальному часі, експресія генів, капілярна кров.